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A novel product of the Duchenne muscular dystrophy gene which greatly differs from the known isoforms in its structure and tissue distribution

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A novel transcript of the Duchenne muscular dystrophy gene has been identified. This 6.5 kb mRNA contains sequences from the 3′ untranslated region of dystrophin mRNA and from the regions coding for the *C*-terminal and the cysteine-rich domains. However, probes for the regions encoding the spectrin-like repeats and the actin-binding domain, as well as probes for the first exons of the muscle- and brain-type dystrophin mRNA, did not hybridize with this new mRNA. Significant amounts of the 6.5 kb mRNA were found in a variety of non-muscle tissues, such as liver, testis, lung and kidney, but not in skeletal muscle. The abundance of this mRNA in the brain is at least as high as that of the previously described 14 kb brain-type dystrophin mRNA.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder resulting in progressive degeneration of the muscles and death in the third decade of life. Some 30% of DMD patients also suffer various degrees of mental retardation. Becker muscular dystrophy (BMD), a less severe disease, is allelic to DMD. The gene which is defective in DMD and BMD is the largest gene known to date, spanning over 2300 kb of the X chromosome. The sequence of its 14 kb mRNA codes for a 3685-amino-acid polypeptide which predicts a rod-shaped protein composed of several domains: an *N*-terminal actin-binding domain, 24 triple-helix repeats similar to those of spectrin, a cysteine-rich domain with two possible Ca^{2+}-binding sites, and a *C*-terminal domain with no sequence similarity with other known proteins [1]. Electron microscopy and immunocytochemical studies have indicated that the protein is associated with the muscle cell membrane [2–6]. It is absent or found in very reduced amounts in DMD patients [2–6]. Early studies suggested that the gene is expressed specifically in muscle tissues [7]. However, using probes for the 5′ region of the mRNA, significant levels of the transcripts were detected in the brain (one-third to one-fifth of the amount in striated muscle) and in the spleen (5–10% of the amount in striated muscle), and smaller amounts were detected in the lung and testis, but not in liver RNA preparations [8–10]. RNAase protection assays and cDNA cloning showed that the first exon of the brain 14 kb DMD mRNA differs from the muscle first exon, indicating the involvement of at least two different promoters in the developmental and cell-type specificities of expression of the gene [9–11].

In this paper we report a new mRNA product of the DMD gene which differs greatly in size, coding content and tissue distribution compared with the known dystrophin mRNAs. This mRNA, which seems to be one of the major products of the DMD gene, is only 6.5 kb long and is found in significant amounts in a variety of non-muscle tissues, including brain, liver, testis, lung and kidney.

MATERIALS AND METHODS

Cell cultures

Primary thigh muscle cell cultures were grown and induced to differentiate as previously described [12]. Human hepatoma cell lines were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum.

Preparation of RNA and RNAase protection assay

RNA was prepared as previously described [13]. Poly(A)-containing RNA was prepared by affinity chromatography on oligo(dT)–cellulose columns (Boehringer) as described by Aviv & Leder [14]. The RNAase protection assays were done as described by Melton *et al.* [15], with minor modifications. The hybridization temperature was 45 °C. For each cRNA probe the digestion by RNAase was done at the temperature which gave the best results (37, 30 or 25 °C). After digestion, the samples were separated on 6% polyacrylamide/urea sequencing gels and the gels were fluorographed at −70 °C

Northern blot analysis

Samples (20 μg) of RNA were size-fractionated on 1% or 0.8% agarose/formaldehyde gels [16]. After electrophoresis, the gels were shaken for 20 min in 50 mM-NaOH, neutralized in 20 × SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate), and blotted on to nitrocellulose filter paper (Schleicher and Schuell). After baking (2 h, 80 °C), the filters were hybridized with the cRNA probe (18 h at 42 °C, in a solution containing 50% formamide, 0.75 M-NaCl, 0.075 M-sodium citrate, 5 × Denhardt solution, 0.05 M-sodium phosphate, pH 6.5, and 100 μg of sonicated salmon sperm DNA/ml). After hybridization the filters were washed for 30 min at room temperature with 2 × SSC/0.1% SDS and twice in 1 × SSC/0.1% SDS at 60 °C for 30 min. The filters were then fluorographed at −70 °C.

Preparation of cRNA probes

The RNA probes used in this study were synthesized on the Gemini 3 (Promega)-based plasmids and contain the following sequences of dystrophin mRNA (from 5′ to 3′). Plasmid MD13 contains a sequence from the 5′ untranslated region of muscle dystrophin cDNA from the cap site to nucleotide 80 (numbering is according to the human dystrophin cDNA sequence [1]). Plasmids MD61 and MD62 contain sequences from the region encoding the actin-binding domain of dystrophin mRNA (nucleo-

Abbreviations used: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; SSC, 0.15 M-NaCl/0.015 M-sodium citrate; 3′UTR, 3′ untranslated region; PCR, polymerase chain reaction.

tides 289–360 and 395–466 respectively). Plasmids MD1, MD51, MD1814 and MD1781 contain sequences from the region encoding the spectrin-like domain (nucleotides 2502–2588, 3680–3758, 7648–7749 and 8894–9020 respectively). Plasmids MD1852 and MD1773 contain sequences from the region encoding the cysteine-rich domain (nucleotides 9420–9626 and 9787–9910 respectively). Plasmid MD1771 contains a sequence from the region coding for the *C*-terminal domain (nucleotides 10401–10552). Plasmids MD3328 and MD1168 contain sequences from the 3′ untranslated region (3′UTR; nucleotides 11451–11541 and 13841–13902 respectively). The construction of plasmids MD13, MD61, MD62, MD1 and MD51 were as described previously [8,9]. The other plasmids were constructed by cloning polymerase chain reaction (PCR)-amplified DNA originating from rat genomic DNA (for the plasmids containing sequences from the 3′UTR), rat liver cDNA, DNA extracted from the human liver cDNA library or human dystrophin cDNA plasmids (obtained from L. Kunkel, Harvard Medical School) to the vector.

RESULTS

In earlier studies we used several probes complementary to sequences in the first 4 kb of the muscle- and brain-type DMD mRNA in RNAase protection assays. These probes were not protected by liver RNA [8–10]. Similar results were obtained using primers from this region in PCR assays. However, when we used probes complementary to sequences in the 3′UTR of the dystrophin mRNA in RNAase protection assays, a significant signal was also obtained with liver RNA. As can be seen in Fig. 1, the probe was protected by total RNA from liver and from two hepatoma cell lines (HepG2 and Huh7). As expected from the previous studies, the probe was protected by RNA from brain and muscle and by RNA from differentiated primary myogenic cultures, but was not protected by RNA from undifferentiated cultures. The sequences which protect the probe were enriched in the poly(A)$^+$ RNA fraction from liver and muscle.

Using additional RNA probes for regions coding for the various domains of dystrophin, the following results were obtained. Probes for the *C*-terminal domain and the cysteine-rich domain were all protected by liver RNA (Figs. 2 and 3). Probes for the region coding for the α-actinin-like actin-binding domain and probes for various regions encoding the spectrin-like repeats were not protected by liver mRNA. All of the probes were protected by muscle and brain mRNA (Figs. 2 and 3). In addition, probes for the first exon of the muscle-type dystrophin mRNA as well as of the brain-type dystrophin mRNA were not protected by liver RNA ([9]; Fig. 3). From the pattern of protection obtained, it was obvious that the liver contains a DMD gene transcript which is very different from the previously described muscle- and brain-type dystrophin mRNAs.

To determine the size and tissue distribution of the liver-type DMD transcript, RNA was extracted from various tissues and from the two hepatoma cell lines and subjected to Northern blot analysis. The blot was hybridized to an RNA probe for the

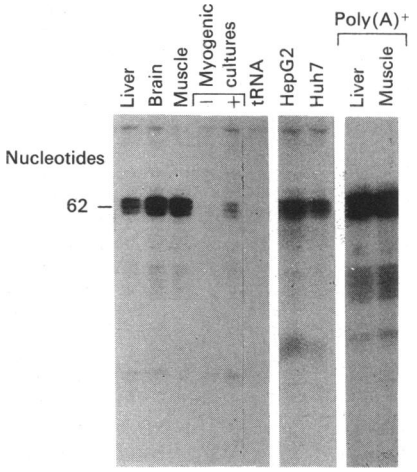

Fig. 1. RNAase protection analysis of the DMD gene transcript

Samples (20 μg) of the indicated RNA preparations were tested with probe MD1168, which is complementary to nucleotides 13841–13902 in the 3′UTR of human muscle dystrophin mRNA. The probe was prepared as described by Melton *et al.* [15]. Hybridization was performed at 45 °C, and digestion by RNAase was done at 37 °C for 90 min.

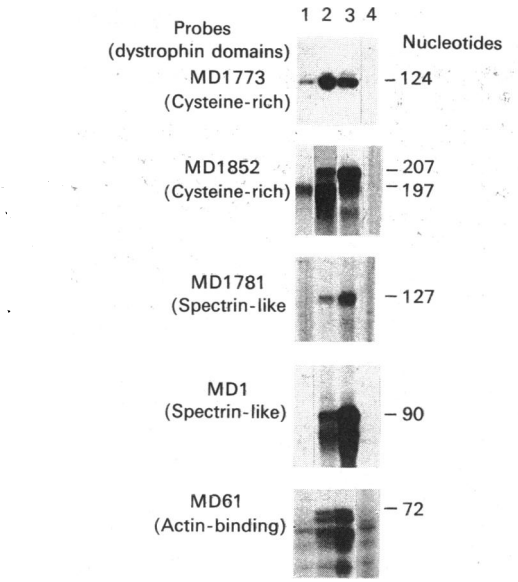

Fig. 2. RNAase protection analysis of DMD gene transcripts with probes for regions of the mRNA coding for different protein domains

Samples (20 μg) of total rat liver RNA (lane 1), rat brain RNA (lane 2), rat muscle RNA (lane 3) and tRNA (lane 4) were tested with the indicated probes. Probes MD1 and MD61 for the spectrin-like domain and for the actin-binding domain were as previously described [8,9]. The other probes and the assay conditions are described in the Materials and methods section. RNAase digestion was performed at 25 °C (MD1773, MD1781), 30 °C (MD1852) or 37 °C (MD1, MD61). The two bands formed with probe MD1852 are most probably due to alternative splicing of the mRNA in this region.

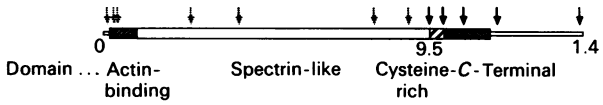

Fig. 3. Schematic presentation of the sequences of the dystrophin 14 kb mRNA which are present or absent in liver RNA

The horizontal bar represents the 14 kb brain and muscle dystrophin mRNAs, the thin bar indicates untranslated regions, and the wide bar is the coding region. The regions coding for the various dystrophin domains are indicated on the bar and described below the bar. Arrows indicate the locations of sequences which were assayed by complementary RNA probes. Solid arrows indicate sequences which protect the complementary probe, when liver RNA was used, and hatched arrows indicate sequences not protected by liver RNA. The probes are described in the Materials and methods section.

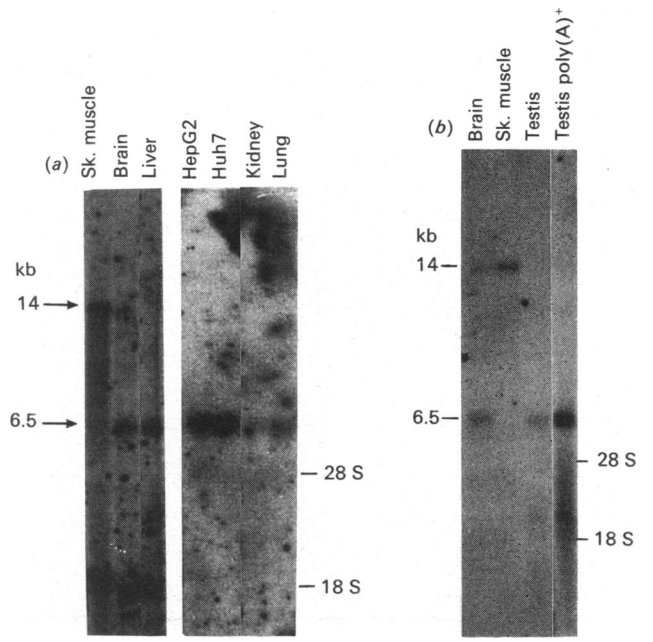

Fig. 4. Northern blot analysis of dystrophin transcripts in rat tissues and human hepatoma cell lines

(*a*) Samples (20 μg) of total or poly(A)$^+$ RNA from the indicated tissue or cell culture were size-fractionated on a 1% agarose/formaldehyde gel. The cRNA probe used for hybridization is a long version of probe MD1168 (complementary to nucleotides 13799–13902 of muscle dystrophin mRNA). The blot was autoradiographed at −70 °C for 3 days. (*b*) Electrophoresis, blotting and hybridization were done as in (*a*), except that a 0.8% gel was used and 15 μg samples were loaded on the gel. Sk. muscle, skeletal muscle.

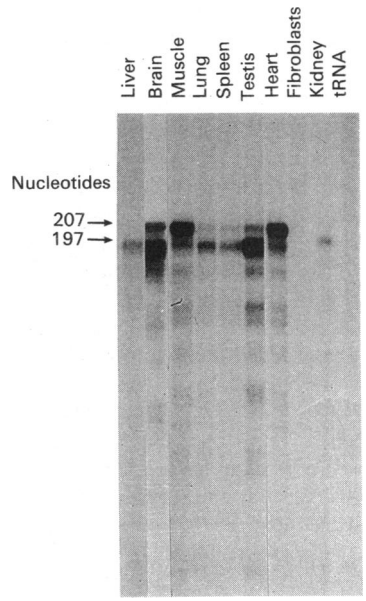

Fig. 5. RNAase protection analysis of the DMD gene transcript in rat tissues

Samples (20 μg) of total RNA from the indicated tissues were assayed with probe MD1852. The hybridization was done at 45 °C and RNAase digestion was done at 30 °C. The gel was fluorographed for 18 h. Assays with samples of RNA from liver, brain and muscle are also presented in Fig. 2.

3′UTR of dystrophin mRNA (complementary to nucleotides 13799–13902). The 14 kb dystrophin mRNA was detected in muscle and brain RNA preparations. In addition, a transcript of about 6.5 kb was detected in RNA preparations from liver, hepatomas, lung, kidney, testis and brain, but not in muscle RNA (Fig. 4). Thus a 6.5 kb DMD gene mRNA is expressed in several non-muscle tissues. Its tissue distribution differs significantly from that of the two 14 kb mRNAs previously described. The reproducible difference in the tissue- and cell-type specificities also shows that this transcript is not a degradation product of the 14 kb transcript.

The presence of substantial amounts of the 6.5 kb transcript in brain mRNA is compatible with the finding that, whereas RNAase protection assays using probes for the 5′ coding regions of the dystrophin mRNA produced weaker signals with brain RNA than with muscle RNA, probes protected by liver RNA produced a signal with brain RNA equivalent to that with muscle RNA (Figs. 1 and 2 and refs. [8–10]).

RNAase protection assays using probes protected by liver RNA confirmed the presence of substantial amounts of transcripts of the DMD gene in many tissues and particularly in testis RNA. The data thus indicate that DMD gene products which differ greatly from the muscle and brain isoforms are present in many tissues. The amounts of these transcripts in some tissues are comparable with the amount of dystrophin mRNA in the muscle (Figs. 4 and 5).

To further characterize the 6.5 kb transcript, a human liver cDNA library (constructed by S. Woo, Baylor College of Medicine, Howard Hughes Medical Center) was screened with probes complementary to distal and proximal regions of the 3′UTR of dystrophin mRNA. (We have chosen the liver library because only sequences from the 3′ end of dystrophin mRNA were detected in this tissue.) Several cDNA clones were isolated and two of them were partially sequenced. The two clones cover most of the 3′UTR of dystrophin mRNA and approx. 700 nucleotides of the 3′ coding region. Over 1400 nucleotides of the two clones were sequenced. Comparison with the published sequence of human skeletal muscle dystrophin [1] revealed mismatches of only two nucleotides. In addition, the exon extending between nucleotides 11221 and 11252 of the published sequence of the muscle isoform (32 bp) was not found in the liver cDNA clone. The occurrence of alternative splicing in this region of dystrophin mRNA was previously described [11].

The identity of the nucleotide sequence in the sequenced parts of the liver transcript with that of the related region in the muscle DMD mRNA (except for the deletion of an exon) shows that the 14 kb and the 6.5 kb mRNAs are transcribed from the same gene. Southern blot analysis using probes for the 3′ end of the mRNA also demonstrated the existence of a single gene (results not shown). These results also rule out the possibility that the liver isoform is transcribed from the recently reported DMD-like gene located on chromosome 6 [17].

Since the 6.5 kb mRNA does not contain the first exons of the muscle or the brain dystrophin mRNA, it is most likely that its transcription is controlled by a third, as yet unidentified, promoter which determines its unique cell-type specificities of expression. The differences between the 6.5 kb mRNA and the 14 kb isoforms occur by alternative splicing and, most probably, alternative initiation of transcription. The existence of more than one 6.5 kb DMD mRNA isoform cannot be ruled out.

DISCUSSION

The present investigation describes a major DMD gene transcript which differs greatly from the known DMD mRNA isoforms in size, structure and tissue distribution.

The data support the notion that the huge DMD locus harbours information for a variety of transcripts which are

expressed in a wide range of tissues, the muscle being just one of them. The isoforms may differ greatly in their tissue specificity, and this is very stringently controlled by a number of different promoters [18].

The localization of dystrophin in the membranes of myofibres and the structure of the protein suggest a structural role for this protein in the muscle. The localization of dystrophin and its biological role in the brain are not yet known. Likewise, the molecular mechanism for the high incidence of mental retardation in DMD-affected children is not yet understood. Our finding that the 6.5 kb DMD gene transcript is expressed in the brain at least at the same level as that of the 14 kb dystrophin mRNA raises the possibility that the absence or misfunction of the protein encoded by this mRNA, solely or together with misfunction of the product of the 14 kb mRNA, is responsible for the high incidence of mental retardation in DMD patients. Since the 6.5 kb mRNA is expressed at relatively high levels in many tissues, it is possible that the absence or malfunction of the protein encoded by this mRNA is the reason for abnormalities found in several cell types of DMD patients (for review see [19]). It is surprising, however, that whereas the misfunction of the DMD gene is so deleterious to muscle, to our knowledge there are no reports of any clear DMD-associated pathology in tissues such as liver and testis in which the amounts of DMD gene transcripts are comparable with those found in muscle.

An additional point of interest is that the muscle dystrophin mRNA, the brain dystrophin mRNA and the 6.5 kb mRNA use the same very long 3′UTR. Comparison of the nucleotide sequences of the 3′UTR of human and chicken dystrophin mRNAs reveals three highly conserved regions, which together cover approximately 900 nucleotides [20]. Such strong conservation indicates an important functional role for those sequences. Since the three mRNAs are differently distributed among tissues and cell types, it is very likely that this sequence conservation is related to a function which is not tissue-specific [21].

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